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14. ABSTRACT
The Six1 homeodomain-containing transcription factor stimulates the proliferation and survival of progenitor cells and plays a role in the epithelial to mesenchymal transition (EMT) during development. Using cell culture and mouse models, we have previously shown that Six1 not only promotes proliferation and survival, contributing to tumorigenesis, but also upregulates the TGF pathway, brings about an EMT-like transformation, and promotes metastasis. There are no known intrinsic activation or repression domains in Six1. The Eya family (Eya1-4), coactivators of Six1, play important roles in Six1-mediated transcriptional activation in development. Here, we preliminarily show that Eya2 is required for Six1-induced upregulation of the TGFβ pathway as well as for most Six1-induced epithelial to mesenchymal transition (EMT) phenotypes. These results suggest that Eya2 is a critical coactivator that is necessary for Six1-induced tumorigenic properties. Thus, as Six1 and Eya are not normally expressed in most adult tissues, the Six1-Eya interaction may be a valuable future therapeutic target for the 50% of primary breast tumors and 90% of metastatic lesions that overexpress Six1.

15. SUBJECT TERMS
Six1, Eya2, Dach, epithelial to mesenchymal transitions (EMT), tumorigenesis, mammary gland, TGF . . -catenin, E-cadherin, metastasis

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INTRODUCTION:

The homeobox transcription factor Six1 is a critical mediator of embryonic development, where it stimulates proliferation and survival of progenitor populations as well as contributes to the epithelial to mesenchymal transition (EMT)[1,2]. Using cell culture and mouse models, we and others have shown that, when expressed out of context in adult cells, Six1 not only promotes proliferation and survival[3,4,5], contributing to tumorigenesis[6], but also brings about an EMT-like transformation which has been linked to metastatic and invasive cancers[6,7]. Six1 has no known intrinsic regulatory domains[1], and as such, is part of the Six-Eya-Dach transcriptional complex. Dach1, co-repressor of Six1, acts in direct opposition to Six1, repressing breast tumor growth and transformation and having reduced expression in metastatic breast cancers[8,9]. The Eya family (Eya1-4), coactivators of Six1, play important roles in Six1-mediated transcriptional activation both in normal development[10,11,12,13,14] and in various diseases where the Six1-Eya1/4 interaction is disrupted[15,16,17,18,19]. Additionally, Eya family members have been found to change Six1-Dach from a repressor to an activating complex[12,20]. Together, these findings suggest that Six1 may require an Eya cofactor to mediate its tumorigenic effects. We are currently analyzing the role of Eya in breast cancer by knocking down Eya levels and asking whether loss of Eya is able to reverse Six1-induced tumorigenic properties. Due to the instability of long term overexpression of Six1 in the MCF12A mammary epithelial cell line, we have moved from this cell line into the MCF7 breast carcinoma cell line for easier initial analysis of the coactivator role of Eya. The MCF7 cells are tumorigenic but non-metastatic. Our lab has shown that overexpression of Six1 in the MCF7 cells induces the TGFβ pathway, properties of EMT, as well as the development of metastasis when orthotopically injected into immunocompromised mice[21]. Aim2 has changed slightly to accommodate Six1-induced phenotypes in the MCF7 cells rather than MCF12A cells but follows the main principles and same timeline originally outlined.

BODY:

Aim 1: Determine whether Eya2 is present in MCF7 cells.

(A) Examine Eya2 protein levels in MCF7-Six1 and MCF7-Ctrl Clones (Months 1-2)

This subaim has been completed. We have examined Eya1 and Eya2 levels in the MCF7-Six1 and MCF7-Ctrl clones as these are the two Eya family members found to significantly correlate with Six1 in human breast cancer patients (as discussed below in Aim3). While Eya1 mRNA was not found to be expressed, Eya2 is expressed at similar mRNA levels in both MCF7-Six1 and MCF7-Ctrls (Fig1). Expression of Eya2, but not Eya1, suggests that Eya2 may be the relevant Eya co-activator for Six1-mediated tumorigensis in breast cancers.

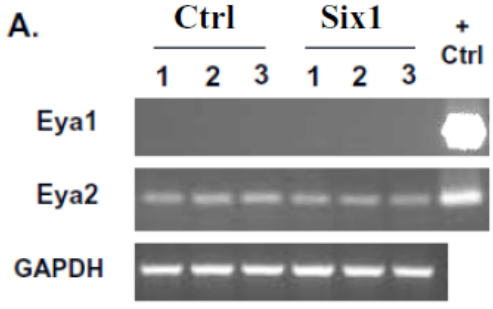

Fig 1. Eya2 is expressed in MCF7-Six1 and MCF7-Ctrl clones. Eya1 and Eya2 expression were analyzed by RT-PCR in MCF7 clones overexpressing Six1 and compared to MCF7 control clones.

(B) Determine if Eya2 interacts with Six1 in MCF7 clones (Months 3-7).
Since it has been well published that Six1 interacts with the Eya family [22], this subaim is not being pursued. Immunoprecipitation of wildtype Eya2 with Six1 does not add any additional information at this time.

Aim 2: Determine the role of Eya2 in promoting Six1-induced tumor progression in MCF7 cells.
(A) Knockdown Eya2 in MCF7 cells overexpressing Six1 (Months 8-9).
This subaim has been completed. We have efficiently knocked-down Eya2 utilizing the SureSilencing system from SABiosciences. 5 Eya2 shRNA constructs and one scrambled negative control, all contained within the pGeneClip vector, were stably transfected into MCF7-Six1 or MCF7-Ctrl cells. Cells were cultured under puromycin selection and individual clones chosen. 2 individual clones from each of two working Eya2 shRNA constructs, as well as 2 scrambled negative control clones, were used to identify the role of Eya2 in Six1-mediated tumor progression (Fig 2). As shown in Figure 2, we have made Eya2 knockdowns in the MCF7-Ctrl line as well as the Six1 overexpressing line to control for any role Eya may play that is not linked with Six1. As we have yet to discover a Six1 independent phenotype in the Eya2 knockdowns, data in the following subaims will only reflect the Eya2 shRNA1 and shRNA2 clones with the MCF7-Six1 parental line as well as the scrambled control in both MCF7-Six1 and MCF7-Ctrl.

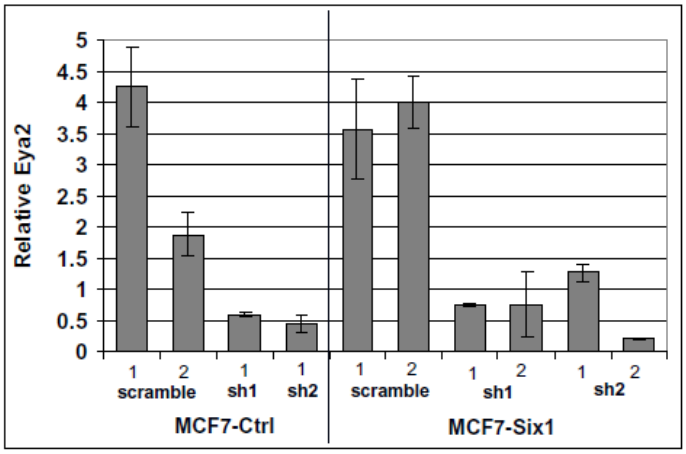

Fig 2. Eya2 is efficiently knocked down in MCF7-Six1 and Ctrl cells. Real Time PCR of Eya2 expression in knockdown and scrambled clones.

(B) Determine if Eya2 mediates Six1-induced TGFβ signaling in MCF7-Six1 cells (Months 10-12).
Six1 overexpression in MCF7 cells activates the TGFβ pathway[21]. We are in the process of analyzing the Eya2 knockdowns to determine if Eya2 is a necessary component for Six1-induced TGFβ signaling. Thus far, we have found that lack of Eya2 reverses TGFβ responsive transcription in the MCF7-Six1 cells back to, or slightly more than, levels detected in the MCF7-Ctrls as measured by 3TP reporter activity (Fig 3). These results suggest that Eya2 is indeed necessary for the ability of Six1 to activate the TGFβ pathway. We are in the process of further analyzing this pathway to determine which stages the Eya2-Six1 complex is necessary. Expression of the TGFβ receptors, as well as activation of the Smad proteins, under Eya2 knockdown conditions are currently being tested.

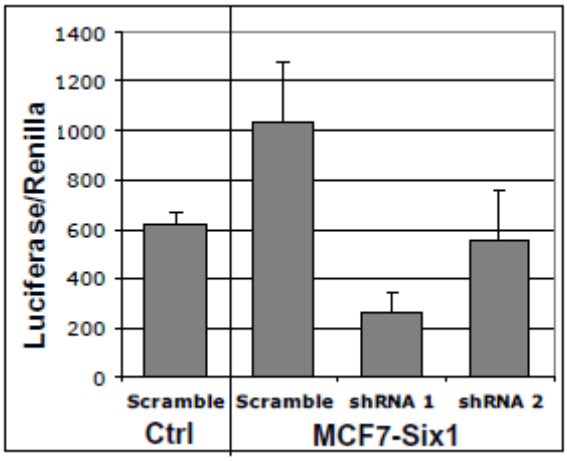

Fig 3. Loss of Eya2 in MCF7-Six1 cells decreases TGF-β responsive transcription as compared to Six1 and Ctrl scramble controls. Responsiveness was tested using luciferase activity of the 3TP reporter construct and normalized to renilla luciferase activity. Data points show the mean of two individual clones from two representative experiments and error bars represent the standard deviation.

(C) Determine if Eya2 mediates Six1-induced features of EMT in MCF7-Six1 cells (Months 13-15)

MCF7 cells overexpressing Six1 acquire mesenchymal properties while losing epithelial ones[21]. Lack of Eya2 in MCF7-Six1 cells reverses the Six1-induced increase in the mesenchymal marker fibronectin (Fig 4a). Eya2 is required for Six1-induced β-catenin responsive transcription as shown by reversal of TOP-FLASH reporter activity in the Eya2 knockdown clones to levels below that of the Ctrl scrambled clones (Fig 4b). Thus far, these data suggest that Eya2 is required for Six1-induced properties of EMT. Work is continuing on this subaim to determine if Eya2 is required for Six1-induced relocalization of β-catenin to the nucleus or if Eya2 is regulating β-catenin responsive transcription through another mechanism.

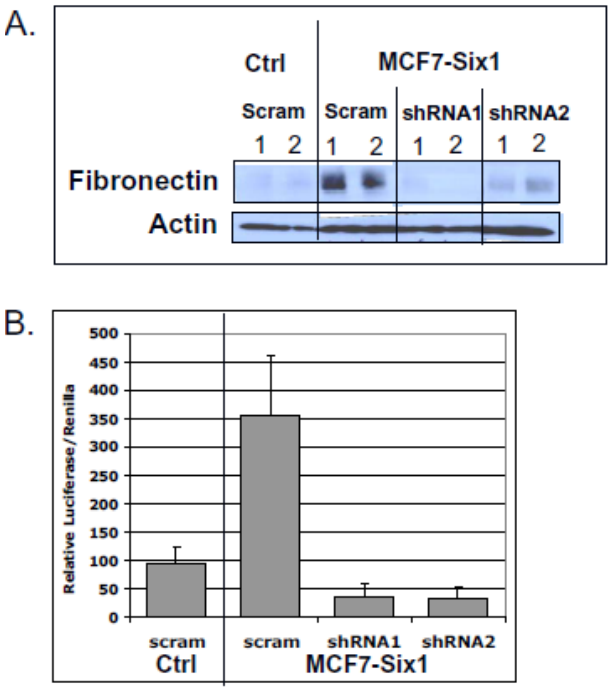

Fig 4. Loss of Eya2 reverses some Six1-induced EMT properties. (A) Western blot analysis was performed on whole cell lysates using Fribronectin and β-actin antibodies. (B) β-catenin induced transcriptional responsiveness was tested using luciferase activity of the TOP-FLASH reporter construct and normalized to renilla luciferase activity. Data points show the mean of two individual clones from two experiments and error bars represent the standard deviation.

In addition to the above mentioned EMT properties, we've analyzed the epithelial marker Cytokeratin 18 (CK18) but found that loss of Eya2 does not reverse the Six1-induced expression of this marker (data not shown). Additionally, Six1 overexpression in MCF7 cells results in a decreased adhesion to different matrixes, a property of a more mesenchymal cell, that is not reversed by loss of Eya2 (Fig 5). These data suggest that either Eya2 is not required for all Six1-induced EMT properties or that some properties, such as decreased adhesion, are more permanent and not reversible merely by interfering with Six1 function. We are in the process of knocking down Six1 in the MCF7-Six1 clones utilizing with pSuperRetro shRNA constructs already available in the to determine which Six1-induced phenotypes are reversible and which are more permanent.

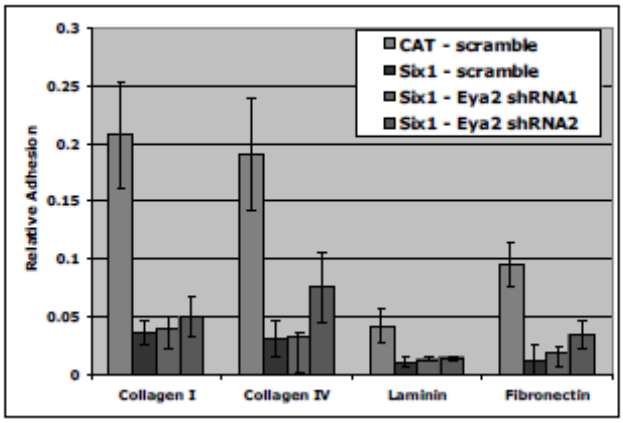

Fig 5. Eya2 is not required for loss of adhesion in MCF7-Six1 cells. Relative cell adherence was measured by crystal violet staining. Data points show the mean of two individual clones from and are representative of two adhesion experiments. Error bars represent the standard deviation.

(D) Determine if Eya2 mediates Six1 induced metastasis (Months 16-27)

Our lab has shown that Six1 overexpressed in the tumorigenic but non-metastatic MCF7 cells induces metastasis when orthotopically injected into immuocrompromised mice, both Nude and NOD/SCID[21]. This aim is ongoing. We have chosen one of the two clones each from shRNA1 and shRNA2 and both clones of the MCF7-Six1 and Ctrl scrambled clones. These clones already contain a ZSGreen fluorescent tag for easier visualization of metastases. The cells were orthotopically injected into the number 4 mammary gland of 10 NOD/SCID mice for each set of clones. When the primary tumors reach $2cm^3$ we will analyze the mice for metastatic burden as well as for location of metastasis. Both the primary tumor and the metastases will be analyzed by immunohistochemistry for Six1 and Eya2 expression as well as epithelial and mesenchymal markers and TGF-β pathway proteins such as Smad3.

Aim3. Determine whether Eya2 is relevant to human breast cancer.

(A) Examine expression of Six1 and Eya2 in human breast tumor samples (Months 28-31).

Not only are Eya2 and Six1 overexpressed in the same cancers[23,24], but additionally they both individually correlate with increased proliferation and shortened patient survival in ovarian cancer[24] suggesting that these proteins may initiate the same protumorigenic pathway. The goal of this aim is to determine if Eya2 is overexpressed in breast cancer and whether it is co-expressed with Six1 by analyzing Clonetech Breast Cancer Profiling Arrays. We have developed our own Eya2 antibody and are in the process of testing this antibody on tissue sections. We will continue with this aim after antibody conditions are worked out.

(B) Correlate expression of Six1 and Eya2 with clinical parameters (Months 32-36).
This subaim will follow after the data for Subaim 3A is obtained.

In the meantime, while troubleshooting antibody conditions for Subaim 3A, we have been mining publicly available microarray data sets in collaboration with Alana Welm at the University of Utah. Together, we have shown that although high levels of Six1 significantly shortens time to metastasis in breast cancer patients, presence of the Eya1/Six1 or Eya2/Six1 transcriptional complex not only further reduces time to metastasis but also significantly decreases survival time in breast cancer patients (Fig 6). Presence of Eya3 and Eya4 do not correlate with Six1. This data in conjunction with the to-date Eya2 knockdown data suggests that Eya2 is a critical cofactor of Six1 in human breast cancer.

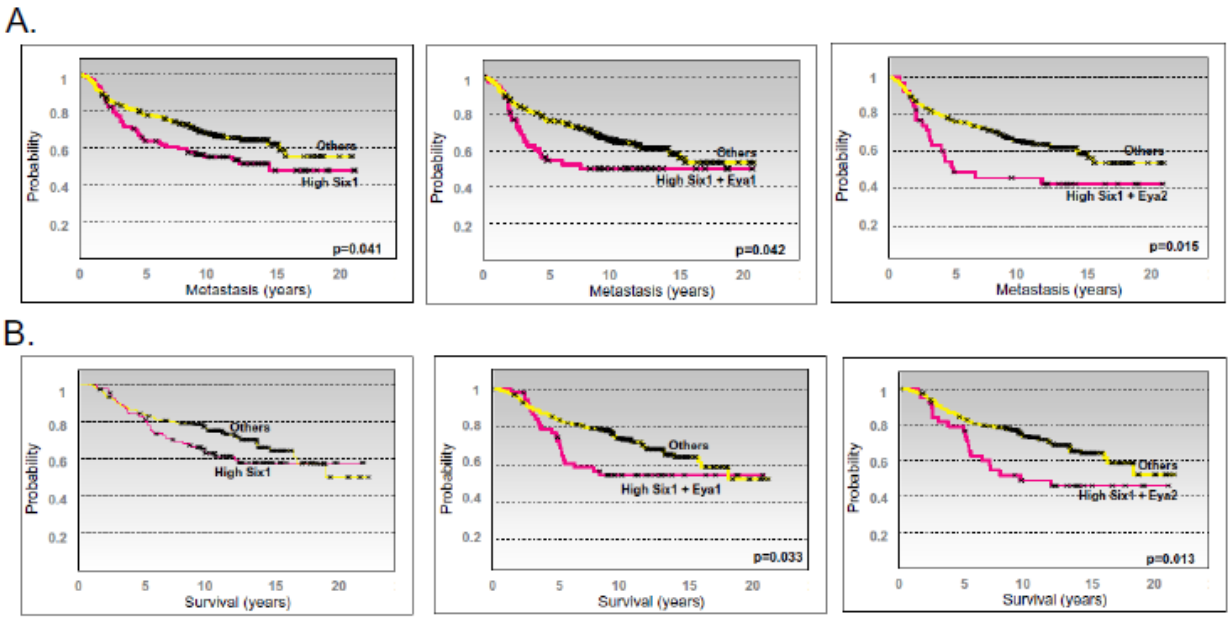

Fig 5. High Six1 with high Eya1 or Eya2 expression correlates with shortened time to metastasis and decreased survival in human breast cancers. In a microarray analysis of 295 women with early stage breast cancer, high Six1 expression correlates with (a) reduced time to metastasis while Eya1/Eya2 expression with high Six1 correlates with an even further reduction in time to metastasis. Additionally, (b) high Eya1/Eya2 with high Six1 expression significantly correlates with shortened survival time while high Six1 alone does not. The median value for Six1 and/or Eya1/Eya2 expression was used to divide the samples into high and low Six1 and/or Eya1/Eya2 expressors.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified that Eya2, but not Eya1, is expressed in MCF7 cells.
- Determined that Eya2 mediates Six1-induced TFGβ responsive transcription.
- Determined that cells overexpressing Six1 require Eya2 for increased expression of the mesenchymal marker Fibronectin.
- Determined that Eya2 mediates Six1-induced β-catenin responsive transcription.

- Determined that loss of Eya2 was not sufficient to reverse the downregulation of the epithelial marker Cytokeratin 18 or the decreased adhesion which are both acquired by Six1 overexpression.
- Determined that high Six1 expression along with high Eya1 or Eya2 expression in early stage breast tumors correlates with an enhanced shortened time to metastasis and statistically significant decreased survival as compared to high Six1 without Eya correlation.

REPORTABLE OUTCOMES:

Research presented in the form of a poster at the Student Research Forum, University of Colorado Denver Anschutz Medical Campus, January 2009. See appendix for poster abstract.

Grants that resulted from this work includes a grant from the Breast Cancer Research Program, Synergistic DOD Idea Award entitled "*Structural and Functional Analyses of the Six1 Transcriptional Complex for Anti-Breast Cancer Drug Design*" (BC084105). PIs: Heide L. Ford and Rui Zhao.

CONCLUSIONS:

The results from studies completed thus far suggest that Eya2 is required for most Six1-induced tumorigenic properties in MCF7 cells. Loss of Eya2 results in a reversal of TGF-β responsive transcription, suggesting that Eya2 may be required for Six1-induced upregulation of the whole TGF-β pathway. In addition, our research suggests that Eya2 is necessary for most Six1-induced properties of the epithelial to mesenchymal transition. It is still unclear as to whether complete reversal of these EMT phenotypes is because Eya2 is not required or whether the phenotypes are just more permanent after initial Six1 overexpression. Overall, thus far, Eya2 appears to be a very important Six1 coactivator that is necessary for Six1-induced tumorigenic properties, suggesting that Eya2 may be a legitimate therapeutic target for the 50% of primary breast tumors and 90% of metastatic lesions that overexpress Six1[3]. As Six1 is overexpressed in a large number of breast tumors and metastases, and not expressed in most adult tissues[3,25], and as Eyas are developmentally restricted, we believe that targeting the Six1-Eya interaction in a clinical setting may successfully treat cancer while limiting side effects. Additionally, we are most interested in future studies with Eya as the Eya family contains phosphatase activity which may be an easier small molecule inhibitor target than the Six1-Eya interaction itself.

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APPENDICIES:

Poster abstract submitted for the Student Research Forum at the University of Colorado Denver Anschutz Medical Campus, January 2009.

The Role of Eya as Cofactors of Six1 in Human Breast Cancer.

S.M. Farabaugh, Alana Welm, H.L. Ford, Department of Biochemistry and Molecular Genetics, University of Colorado Denver Anschutz Medical Campus, Aurora, CO.

The Six1 homeodomain-containing transcription factor stimulates the proliferation and survival of progenitor cells and plays a role in the epithelial to mesenchymal transition (EMT) during development. Using cell culture and mouse models, we have shown that, when expressed out of context in adult cells, Six1 not only promotes proliferation and survival, contributing to tumorigenesis, but also upregulates the TGFβ pathway, brings about an EMT-like transformation, and promotes metastasis. Most importantly, reducing Six1 levels decreases cancer cell proliferation and metastases in several models of cancer, strongly suggesting that Six1 will make an excellent cancer therapy target. There are no known intrinsic activation or repression domains in Six1. The Eya family (Eya1-4), coactivators of Six1, play important roles in Six1-mediated transcriptional activation in development. The fact that Eyas promote cellular proliferation through Six1, that the Six1-Eya interaction is lost in several diseases, that both Eya and Six1 are implicated in the same cancers, that Eya is needed to revert Six1 and its corepressor Dach to an activating complex, and that Eya phosphatase activity is required for Six1 activation, suggests that Eya may be a relevant co-activator for Six1-induced breast tumorigenesis. Here we show that while Six1 correlates with shortened time to metastasis in breast cancer patient tumors, presence of both Eya2 and Six1 together further reduces time to metastasis as well as significantly correlates with decreased survival. Additionally, decreasing Eya2 levels in MCF7 cells overexpressing Six1 reverses Six1-induced upregulation of the TGFβ pathway and most Six1-induced EMT phenotypes, suggesting that Eya2 is required for the ability of Six1 to mediate tumor progression. As Eya2 appears to be required for Six1 activity in breast cancers and as Six1 and Eya are not normally expressed in most adult tissues, we believe that targeting Eya phosphatase activity or the Six1-Eya interaction with small molecule inhibitors could result in new chemotherapeutics for breast cancer patients.